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Canine Distemper Virus Seroprevalence in Domestic Cats and Identification of the Feline Signaling Lymphocyte Activation Molecule

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1 Zusammenfassung

Das Staupevirus (Canine distemper virus, CDV) verursacht eine schwerwiegende multisystemische Erkrankung bei Kaniden und anderen Karnivoren. Seit dem Löwen-Massensterben 1994 im Serengeti Nationalpark in Tansania ist unumstritten, dass auch Feliden infiziert werden können. Im Gegensatz zur Situation bei den Grosskatzen ist die Bedeutung der Staupeinfektion bei Hauskatzen aber grösstenteils unbekannt. In *in vitro* Versuchen konnten wir zeigen, dass sich der Staupevirusstamm A75/17 gleich gut in Hunde- wie auch in Katzen-Lymphozyten vermehrt. In diesem Zusammenhang haben wir den Morbillivirus-Rezeptor SLAM (Signaling Lymphocyte Activation Molecule), der zuvor erst in anderen Spezies bekannt war, mittels PCR neu auch in Katzen-Lymphozyten nachgewiesen. In einem zweiten Schritt wurden ein indirekter Immunfluoreszenz und ein Western-Blot Test für die schnelle Erkennung von CDV-spezifischen Antikörpern in Katzenseren entwickelt. Antikörper gegen das Hämagglutinin- und Nukleokapsid-Protein vom Staupevirusstamm A75/17 existierten sowohl in Blutproben von Hauskatzen aus der Schweiz (10/500, 2%), wie auch aus Spanien (8/97, 8.2%) und den Vereinigten Arabischen Emiraten (17/93, 18.3%). In den beiden letzteren Ländern war der Anteil an Katzen mit Antikörpern gegen CDV in der FIV (Felines Immunschwäche Virus)-seropositiven Population signifikant grösser als in der FIV-seronegativen Population ($P = 0.0001$). Es wurde jedoch kein Zusammenhang zwischen der Seropositivität und dem Auftreten von klinischen Symptomen gefunden. Nur ein sehr kleiner Anteil der seropositiven Katzen hatte auch virusneutralisierende Antikörper gegen A75/17 und/oder den Onderstepoort CDV-Stamm. Von den 72 getesteten Grosskatzen aus europäischen Zoos wiesen 7 gesunde ungeimpfte Tiere Antikörper gegen Staupe auf, 2 davon auch virusneutralisierende. Zusammenfassend lässt sich sagen, dass die Staupevirusinfektion bei Katzen weit verbreitet ist ohne zu klinischer

Symptomatik zu führen. Die Rolle der Hauskatze als Träger des Staupevirus muss deshalb in weiteren Studien untersucht werden.

2 Summary

Canine distemper virus (CDV) causes fatal disease in many species of carnivores, including felids. In contrast to the situation in large felids, the importance of CDV infection in domestic cats remains largely unknown. In this study, we showed that CDV strain A75/17 replicated as efficiently in feline as in canine peripheral blood mononuclear cells in vitro. The presence of the morbillivirus receptor signaling lymphocyte activation molecule (SLAM) was demonstrated by PCR in feline cells. An indirect immunofluorescence and a Western blot assay were established for the rapid detection of CDV-specific antibodies in cat sera. Antibodies against the hemagglutinin and nucleocapsid proteins of CDV strain A75/17 were present in domestic cats from Switzerland (10/500, 2%), Spain (8/97, 8.2%) and the United Arab Emirates (17/93, 18.3%). The frequency of CDV seropositive cats in Spain and the United Arab Emirates was significantly higher in the feline immunodeficiency virus (FIV) seropositive population than in FIV negative cats (P = 0.0001). No clinical signs were found in most CDV seropositive cats. Antibodies neutralizing A75/17 and/or the Onderstepoort strain of CDV were found in only a small proportion of seropositive cats. Among the 72 tested captive large felids from Europe, 7 healthy non-vaccinated animals were found to be CDV seropositive and 2 of them had also virusneutralizing antibodies. Based on these data, we conclude that CDV infection in domestic cats is widespread, although the infection remains subclinical. Therefore, the role of the domestic cat as a carrier of CDV needs to be further investigated.

3 Introduction

Canine distemper virus (CDV), a member of the genus Morbillivirus of the family *Paramyxoviridae*, causes serious multisystemic disease in canids worldwide (5). Due to regular vaccination with modified live virus vaccines, the incidence of clinical distemper in domestic dogs has decreased considerably during the last decades. However, several outbreaks of the disease, also in vaccinated dogs, raised questions about the efficacy of the vaccines (8, 13, 16, 25). In addition, CDV has a broad host range, which makes eradication nearly impossible. CDV infections have been diagnosed in terrestrial and marine mammals worldwide (7). Members of all families of terrestrial carnivores were shown to be susceptible to the infection: *Canidae*, *Mustelidae*, *Hyaenidae, Procyonidae*, *Ursidae*, *Viverridae* and also *Felidae* (12).

In 1994, CDV caused an unexpected mass mortality in the lion population of the Serengeti National Park in Tanzania, resulting in the death of around 1000 lions (29). Earlier reports of clinical distemper in captive large felids in the United States (4, 9) and a retrospective study based on necropsy cases of captive lions and tigers in Switzerland (26) revealed that CDV infection in large felids has been present for a longer time and is more widespread than previously thought. However, the importance of CDV in domestic cats remains unclear. Specific-pathogen-free cats have been shown to be susceptible to experimental infection (2, 18). Interestingly, despite a slight increase in body temperature, no clinical signs were observed. A seroprevalence study carried out in the Asian countries Taiwan, Vietnam and Japan, indicated different prevalences of CDV-specific antibodies in domestic cats, varying from 0% to 88.8%, depending on the region studied and on exposure to dogs (19). Acutely infected dogs shed virus in all body excretions, regardless of clinical signs. The main route of transmission is via aerosol of respiratory secretions. Virus transmission from dogs to cats, but not vice

versa, was documented experimentally (2). There was no correlation between CDV seropositivity and the clinical status in the cats from Asia.

The aim of this study was to determine the seroprevalence of antibodies to CDV in domestic cats in countries harbouring different population densities of feral dogs and cats and to possibly correlate these findings with the presence of circulating distemper in dogs. We were also interested in establishing an association between the seropositivity in cats and the existence of clinical signs of disease. In addition, in vitro infectivity assays were performed to compare the efficiency of CDV infection in feline and canine cells. Due to the fact that CDV readily replicated in feline peripheral blood mononuclear cells, the feline analogue of the signaling lymphocyte activation molecule (SLAM), a morbillivirus receptor, was identified and partly sequenced. A better understanding of the CDV infection in domestic cats may be an important aspect for the health management of both cats and dogs as cats could either be affected by the disease themselves or play a role as carriers.

4 Materials and Methods

4.1 Cells and virus strains

Vero cells were grown in cell culture medium (RPMI-1640, Sigma-Aldrich, Buchs, Switzerland) supplemented with 10% heat-inactivated fetal calf serum (FCS, BioConcept, Allschwil, Switzerland), 2 mM L-glutamine (Gibco, Eggenstein, Germany), 100 units/ml penicilline G, 100 µg/ml streptomycin and 0.25 µg/ml amphotericine B (Gibco). Peripheral blood mononuclear cells (PBMC) were isolated from freshly collected blood of specific-pathogen-free (SPF) dogs and cats. Cats originated from Liberty Research (Waverly, NY) and dogs from RCC Ltd (Füllinsdorf, Switzerland). The blood collection was

carried out by venipuncture using K3-EDTA-anticoagulated vacutainers. PBMCs were isolated by Ficoll gradient centrifugation (Histopaque-1077, Sigma-Aldrich) and grown in cell culture medium mentioned above, supplemented with 1 mM β-mercaptoethanole and 10 µg/ml concanavalin A (both Sigma-Aldrich). After 24 h 100 units/ml of human interleukin-2 (kindly provided by Novartis AG, Basel, Switzerland) were added and used for all further medium replacements.

The Onderstepoort (OP) CDV vaccine strain was propagated in Vero cells, using techniques described by Appel and Robson (1), with the following modifications: Vero cells were inoculated at 70 – 80% confluency with 5 ml of virus suspension containing 10^4 tissue culture infectious dose 50 ($TCID_{50}$) in 75 cm^2 cell culture flasks. After virus adsorption for 2 h at 37°C, 15 ml of cell culture medium supplemented with 2% FCS was added per flask and cultures were incubated until a pronounced cytopathic effect was observed. The virulent strain A75/17 from lymphoid tissue of experimentally infected dogs (11) was passaged once in PBMCs from a SPF dog as previously described (3) before using for infection.

4.2 Infection of feline PBMCs with A75/17

Supernatant of A75/17 infected canine lymphocytes was centrifuged for 10 min at 2000 x g and diluted 1:4, 1:16, 1:64 and 1:256 with RPMI-1640. Two hundred microliters of each dilution were used for inoculation of 10^6 PBMCs collected from either a SPF dog or a SPF cat using a 24-well plate. Additionally, 200 µl of each dilution were cultured without cells and used as „virus only“ control. Uninfected cells were cultured in parallel as negative controls. One hour after infection, 800 µl of fresh medium was added to each well. Two hundred and fifty microliters of each cell-suspension were collected daily and replaced by fresh cell culture medium. Infected and non-infected

PBMC were analyzed by indirect immunofluorescence assay (IFA) and the supernatants were tested for the presence of CDV-RNA by real time TaqMan reverse transcriptase polymerase chain reaction (RT-PCR) on days 1, 2, 3, 4, 6, 8 and 10 post-inoculation. For this purpose, total RNA was isolated from 200 µl of supernatant using MagNaPure LC Total Nucleic Acid Isolation Kit (Roche Diagnostics, Rotkreuz, Switzerland) according to the manufacturer's recommendations and RT-PCR was performed with a primer set specific for the morbillivirus polymerase gene as previously described (6). PCR was run for 45 cycles and reactions giving no positive fluorescence signal after 45 cycles were judged negative.

4.3 Cloning and sequencing of the feline signaling lymphocyte activation molecule (SLAM)

Total RNA was isolated from 10^6 feline infected PBMCs (see above) using the RNeasy Mini Kit (Qiagen, Hombrechtikon, Switzerland) according to the manufacturer's protocol.

Based on consensus sequences from human, bovine and canine SLAM degenerated primers were designed to amplify a 458 bp-long stretch of the gene. The forward primer cwSLAM.427f 5'-GCT YTA TGA RCA GGT CTC CAC TCC-3' and the reverse primer cwSLAM.884r 5'-TCT GGA CTT GGG CAT AGA TVG TAA G-3' were used to amplify 5 µl of RNA using the SuperScript™ III One-Step RT-PCR System (Invitrogen, Basel, Switzerland), 0.2 µM of each primer, in a total volume of 25 µl. PCR cycling conditions were as follows: 30 min at 60°C (RT step), 2 min at 94°C, 40 cycles of 15 sec at 94°C (denaturation), 30 sec at 50°C (annealing) and 1 min at 68°C (extension), followed by 5 min at 68°C.

The resulting PCR band was analyzed on 2% agarose gels; the amplicon was purified using the MinElute Gel Extraction Kit (Qiagen). The

purified PCR product was cloned into the TOPO TA™ cloning vector (Invitrogen). The clonal DNA was checked for insert by digesting the DNA with the restriction enzyme *Eco*RI. Positive clones were then sequenced from both sides. Cycle sequencing was performed with approximately 20 ng of DNA and 3.3 pmol plasmid-specific primers using the BigDye Terminator Cycle Sequencing Ready Reaction Kit v1.1 (Applied Biosystems, Rotkreuz, Switzerland). Cycling conditions were as follows: 1 min at 96°C, then 25 cycles at 96°C for 10 sec and 50°C for 5 sec, followed by 60°C for 4 min. Products were purified using the DyeEx Spin column (Qiagen), and analyzed on the ABI Prism 310 Genetic Analyzer (Applied Biosystems). The sequences of two independent clones were aligned to one consensus sequence by SeqScape (Version 1.1, Applied Biosystems) and then compared to reference sequences deposited in the genebank.

4.4 Immunization of two cats with an OP-mlv-vaccine

To produce antibodies to CDV as positive control 2 one-year old cats were immunized subcutaneously three times with two weeks intervals using a commercially available OP modified-live virus vaccine (Vetamun Standard, Veterinaria, Zurich, Switzerland). Blood was collected twice a week and analysed for the presence of both CDV RNA by real time RT-PCR and antibodies by IFA. The serum collected at week three after the last vaccination was used as a positive control for the immunofluorescence and Western blot assays.

4.5 Serum samples

Serum samples collected from Swiss domestic cats (n = 500) consisted of 208 samples of healthy and 292 samples of ill cats, which were collected at clinical practices and animal shelters between June and September 1996 or at the Veterinary Hospital of the Vetsuisse Faculty, University of Zurich, Switzerland in 2005. Samples of Spanish domestic cats (n = 97) were collected in 2005 at animal shelters, clinical practices and the Madrid zoo, where many feral cats live inside the zoo facilities. The samples originating from the United Arab Emirates (n = 93) were obtained from feral cats, captured in Abu Dhabi in 1999 in the context of a project for public health management.

Serum samples originating from captive large felids (n = 72) were collected from healthy and ill lions (*Panthera leo,* n = 21), tigers (*Panthera tigris,* n = 22), ligers (*Panthera leo* x *tigris,* n = 2), leopards (*Panthera pardus,* n = 9), snow leopards (*Uncia uncia,* n = 11) and clouded leopards (*Neofelis nebulosa,* n = 7) between January 2001 and August 2005 in Switzerland, Germany and Austria.

4.6 Indirect immunofluorescence assay

Vero cells expressing the nucleocapsid (N) protein of A75/17 were used to establish an indirect IFA. Subconfluent Vero cells were transfected with the eukaryotic expression plasmid pCI (Promega, Wallisellen, Switzerland) containing the cDNA of the CDV N protein (11) using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA) as recommended by the manufacturer. Cells were trypsinized after 48 h, washed with 1x HBSS (Gibco), spotted onto 12-well glass slides and air-dried. Onto every second well, non-transfected cells were spotted as a negative control. After fixation of the cells with ice-cold acetone the slides were stored at -20°C until use.

Cat serum samples were diluted 1:20 in phosphate-buffered saline (PBS, 150 mM NaCl, 8 mM Na_2HPO_4, 2 mM NaH_2PO_4, pH 7.4). Fifteen microliters of each sample were placed onto a well with either transfected or non transfected cells and the slides were incubated for 1 h at 37°C in a moisturized chamber. The slides were subsequently washed three times with PBS. Fluorescein isothiocyanate (FITC)-conjugated goat anti-cat IgG (Jackson ImmunoResearch, Cambridgeshire, UK) was diluted in PBS (1:400) and used as a secondary antibody. After 1 h of incubation at 37°C the slides were again washed three times with PBS and Vectashield Mounting Medium with DAPI (4',6-Diamidino-2-phenylindole, Vector Laboratories, Burlingame, CA) to counterstain the DNA was used for embedding. The slides were examined under a fluorescence microscope and serum samples, which resulted in a fluorescent signal with the transfected but not with the non transfected cells, were considered positive for the presence of antibodies specific for the N protein of CDV or related morbilliviruses.

4.7 Western blot analysis

For antigen production canine PBMCs infected with the CDV strain A75/17 were cultured for 3 weeks in 1 L of cell culture medium as mentioned above. Cells were removed by centrifugation at 6'000 x g for 20 min. After careful harvesting of the cell culture supernatant, the virus was pelleted by centrifugation (160'000 x g, 50 min) using a Sorvall T647.5 rotor and resuspended in 5 ml TNE-buffer (10 mM Tris, 100 mM NaCl, 1 mM EDTA, pH 7.5). This sample was again centrifuged for 4 h at 80'000 x g in a Sorvall AH629 rotor through a 20% (w/w) sucrose layer onto a cushion of 60% (w/w) sucrose. The band at the interface was collected and virus was again pelleted by centrifugation as described above. All centrifugation steps were performed at 4°C.

The viral pellet was used for the preparation of the Western blot membranes. Viral proteins were fractionated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose transfer membranes (22). Each gel also contained a protein standard (Precision Plus Protein Dual Color Standards, Bio-Rad, Hercules, CA). The membranes were cut into strips of 2 mm, which were incubated either with the monoclonal antibody (mAb) D110 specific for the nucleocapsid protein (1µg/ml) (10), XI-23 specific for the hemagglutinin (H) protein (1µg/ml) (11) or cat serum samples at a dilution of 1:1000. Samples were kept at room temperature overnight. After two washing steps using Tween wash buffer (150 mM NaCl, 0.05% Tween 20, pH 7.4) the strips were incubated for 2 h with the secondary antibody (peroxidase-conjugated rabbit anti-mouse IgG or goat anti-cat IgG, Jackson ImmunoResearch, diluted 1:1000). Following another washing step to remove unbound secondary antibody conjugate, 4-chloro-1-naphtol was applied directly to the strips for color development. The reaction was stopped after 5 min by adding destilled water.

4.8 Serum neutralization test using CDV strain OP

Microneutralization assays were performed as previously described (1). Briefly, serum samples were heat-inactivated at 56°C for 30 min and 200 µl of the ten-fold diluted samples were mixed with 200 µl of a virus suspension containing 100 $TCID_{50}$ of CDV OP. After an incubation of 60 min at 37°C 100 µl of the mixtures were added in quadruplicate to approximately 80% confluent Vero cells in a 96-well plate. Three days after cultivation, the cells were examined microscopically for the development of typical cytopathic effects. Virus-neutralizing antibody titers were calculated by the Spearman-Kärber method (15).

4.9 Serum neutralization test using CDV strain A75/17

Serum samples were heat-inactivated at 56°C for 30 min and diluted 1:25 with cell culture medium. Two hundred and fifty microliters of each sample plus 4 „medium only“ controls were mixed with 250 µl of an A75/17 virus suspension and incubated for 60 min at 37°C. Subsequently, 400 µl of these mixtures were given to 10^6 feline PBMCs in 24 well plates. After 2 hours of incubation 600 µl of fresh cell culture medium was added. Supernatants were analysed for the presence of CDV-RNA by real time TaqMan RT-PCR on days 3 and 6 post-infection (pi). If the Ct value difference of a sample compared to the mean Ct value of the 4 „medium only“ control cultures on day 6 pi was greater than 3, the serum was considered positive for the presence of virusneutralizing antibodies. Using serum of 10 SPF cats as a negative control the Ct value difference was never greater than 0.6.

4.10 FIV enzyme linked immunosorbent assay and western blot

Cat serum samples were analysed for the presence of antibodies against feline immunodeficiency virus (FIV) using recombinant transmembrane antigen in an enzyme linked immunosorbent assay (ELISA) as previously described (23). Weak positive results were confirmed by Western blot analysis (22).

4.11 Statistical analysis

To determine whether a relationship between CDV and FIV seropositivity existed, we performed contingency table analysis by use of the software StatView (Version 5. SAS Institute Inc., Cary, NC). P-values <0.05 were considered as significant.

5 Results

5.1 Infection of cat PBMC with A75/17 in vitro

In order to compare the susceptibility of canine and feline PBMCs towards CDV, we performed an in vitro infectivity assay using PBMCs of the two different species and different virus dilutions. Feline PBMCs were as susceptible to CDV infection in vitro as canine PBMCs. Indirect IFA using the mAb D110 specific for the N protein revealed only few fluorescent cells on day 1 pi. However, on day 3 pi most of the canine and feline cells that were incubated with the non-diluted virus tested positive in the IFA. The number of fluorescent cells was proportional to the virus dose used for inoculation. The virulent CDV strain A75/17 replicated readily in both dog and cat PBMCs, which was demonstrated by the almost identical viral copies found to be present in the supernatant of dog and cat lymphocyte cultures over time (Fig. 1). The signals obtained by real-time RT-PCR reached comparable cycle threshold (Ct) values in canine and feline cell cultures demonstrating that there is no significant difference in lymphocyte susceptibility to CDV infection *in vitro* between dog and cat.

5.2 Cloning of feline SLAM

In view of further functional experiments we decided to isolate, clone and sequence the feline lymphocyte signaling molecule. Based on the consensus sequences from human, bovine and canine SLAM and using degenerated primers we succeeded in amplifying part of the feline SLAM gene from feline infected PBMCs. The cloned sequence was 458-bp long. We sequenced two independent clones. Both sequences were identical. Identities

at the nucleotide and protein level with sequence deposited in the Genbank are depicted in Table 1.

An alignement of feline and canine SLAM amino acid sequence is shown in Figure 2.

5.3 Indirect immunofluorescence assay

We aimed to establish an indirect IFA for the rapid detection of antibodies against the N protein of CDV in cat sera. To this end, we transfected Vero cells with a plasmid containing the cDNA coding for the N protein of CDV strain A75/17. Incubation of the transfected cells with the mAb D110 against the N protein showed cytoplasmatic fluorescence in about every twentieth cell whereas no fluorescence could be observed in the non-transfected control cells (Fig. 3). Furthermore, the slides were evaluated using CDV-antibody positive samples originating from lions from the Serengeti National Park and from CDV vaccinated cats. Of the 10 lion sera, 6 had been shown to be seropositive in an earlier serum neutralization assay (29). The same 6 samples showed a strong fluorescence when the transfected cells were used. The sera of the 2 CDV immunized cats tested negative in the IFA before vaccination, but already two weeks after the first immunization, a strong fluorescent signal could be observed. From these observations it was concluded that CDV-transfected Vero cells can readily be used in an indirect IFA for the detection of anti-CDV N protein antibodies.

5.4 Western blot analysis

To confirm the IFA results by an additional and independent method we established a Western blot assay using denatured proteins of CDV strain

A75/17 as antigens. Incubation of the Western blot strips with the mAb XI-23 against the H protein resulted in one sharp band at the molecular weight of approximately 70 kDa according to the protein standard (Fig. 4). Several bands were found on strips incubated with the anti-N protein antibody D110. As previously shown, these bands represent protein degradation products (27). The strongest band among them, at the molecular weight of approximately 60 kDa, was also found on strips incubated with serum of the CDV immunized cats and lions from the Serengeti National Park with clinical distemper (Fig. 4). Serum samples that recognized bands at the height of those recognized by the mAbs, indicating a reaction with the H and N protein of CDV, were considered positive in the Western blot assay. Serum samples were diluted 1:1000 in order to eliminate non-specific bands found at lower dilutions in sera from SPF cats.

5.5 Detection of antibodies against CDV in domestic cats from Switzerland, Spain and the United Arab Emirates

The main goal of this study was to determine the prevalence of antibodies against CDV in domestic cats in different countries and to elucidate a possible correlation between seropositivity and clinical signs of disease. Cat sera were screened for antibodies against the CDV N protein in a 1:20 dilution by immunofluorescence. Samples that tested positive in the IFA were tested by Western blot to confirm the results. Only cat sera, which were both fluorescent positive and showed the two typical bands on the Western blot strips, were considered to be truly seropositive. According to these criteria 2% (10/500) of the samples from the Swiss domestic cats were seropositive. These samples were additionally analysed for virus neutralizing antibodies against the CDV strains OP and A75/17 (Table 2). Of note, there was no

correlation between the clinical status of the tested cats and the seroprevalence.

Among the tested cats from Spain 8.2% (8/97) were found to be CDV seropositive. Two of these cats had also virus neutralizing antibodies. The highest number of seropositive cats existed in Abu Dhabi, United Arab Emirates (17/93, 18.3%). But despite clear fluorescent signals and broad bands in the Western blot, none of these cats had virusneutralizing antibodies. Additional information about the presence of antibodies against different viruses as feline leukemia virus, feline immunodeficiency virus, feline corona virus, feline herpes virus and feline calici virus existed for the samples from Abu Dhabi. Remarkably many cats that were CDV seropositive had also antibodies against FIV. Therefore, serum samples from Switzerland and Spain were also analysed for the presence of FIV-specific antibodies. The number of FIV seropositive animals in Switzerland was too low for an analysis. Among the 25 CDV seropositive sera from Spain and the United Arab Emirates 10 samples tested positive for the presence of FIV-specific antibodies by enzyme-linked immunosorbent assay and Western blotting. To determine whether a correlation between FIV and CDV seropositivity existed, we performed a contingency table analysis. The proportion of CDV seropositive cats was higher in the FIV seropositive population (0.36) than in the FIV seronegative population (0.09). The difference in proportions was significant, when chi-square analysis was performed, $\chi^2(1, N=190)=14.622$, $P=0.0001$.

5.6 Detection of antibodies against CDV in captive large felids from European zoos

In contrast to CDV infection in domestic cats, CDV infection in lions and tigers is well-known. However, there are only few data about the CDV seroprevalence in captive large felids. The CDV-seropositive rate in the 72

captive large felids in Swiss, Austrian and German zoos was 9.7%. Two tigers, 2 ligers and 3 clouded leopards which, to our knowledge, had never been immunized against CDV were found to be seropositive. The 2 ligers were also positive in the serum neutralization assays. FIV-specific antibodies were found in 11.1% of the captive large felids. However, none of the CDV-positive animals tested positive for the presence of antibodies to FIV.

6 Discussion

Since the outbreak of clinical distemper in the Serengeti lion population in 1994, it is widely accepted that also felids can be affected by CDV infection. However, despite the huge population size of domestic cats and their close contact to domestic dogs, there is only limited information on CDV infection in this species. Experimentally infected SPF cats in two different experiments produced virus neutralizing antibodies and showed no clinical signs. Infectious virus was isolated from lymphatic and lung tissues of intranasally infected cats between days 5 and 7 pi in one experiment (2), using the Snyder Hill strain for infection. In an other experiment, cats were infected intratracheally with CDV field strain A92-27/4, an isolate from a diseased leopard, and infective virus was isolated from PBMCs between days 1 and 21 pi (18). In both experiments, using either a vaccine or a field strain of CDV, infection in domestic cats proceeded like infection with an attenuated CDV strain in dogs. The reason for this phenomenon is unknown. In our study, we were able to show that CDV field strain A75/17 replicated as efficiently in feline PBMCs in vitro as in canine PBMCs. There was no obvious difference between the two species in this in vitro infectivity assay. Therefore, the difference in the susceptibility to CDV infection between dogs and cats does not seem to be caused by any PBMC receptor differences. On the contrary, our results indicate the presence of a feline signaling lymphocyte activation molecule (SLAM, CD150). SLAM is a

membrane glycoprotein found in human beings, monkeys, dogs, cows, rats and mice and known to act as a cellular receptor for different morbilliviruses (33, 34). It is expressed on activated lymphatic cells and the only known receptor for CDV field strains at the moment (30). We were able to sequence a 458-bp long stretch of the feline SLAM gene with 84% identity to the canine sequence.

Natural CDV infection in domestic cats was demonstrated by the presence of virus neutralizing antibodies in cat sera collected in Asian countries (19). The seroprevalence was dependant on the region studied and on exposure to dogs. In our study, seropositivity was determined by both immunofluorescence and Western blot assays. Antibodies of seropositive cats reacted with the N protein of CDV strain A75/17 expressed by transfected Vero cells used in the IFA and with the denatured N and H protein of A75/17 on Western blot strips. Several bands representing protein degradation products were found on Western blot strips incubated with the mAb D110 specific for the N protein. In contrast, only one strong band was seen in Western blot strips incubated with feline serum samples. This can be explained by the much higher concentration of N specific antibodies using the mAb compared to the thousandfold diluted cat serum samples. According to these criteria, we found a low seroprevalence in domestic cats in Switzerland, which can be explained by the low incidence of distemper in dogs in this country. Due to dog registration in the local communities and capturing of stray dogs by animal welfare organizations, only few feral dogs live in Switzerland. In addition, most of the dogs receive at least their first two vaccinations, which confer immunity for 3 years (17). This protects dogs during the most critical age of 3 to 6 months. In addition, morbilliviruses do not persist in an infectious form in the environment. They rely on a constant availability of infected shedders and susceptible hosts. Thus, distemper is rarely occurring in the Swiss dog population.

Serum samples from Switzerland originated from the whole country whereas samples from Spain and the United Arab Emirates were collected mainly in one region of the country and were therefore less representative. Nevertheless, there was a relatively high rate of CDV seropositive cats in Spain which could be explained by the large number of feral dogs and the relatively low vaccination rate in this country. As a consequence, there are a high number of carriers, which keep infectious virus in the population and are able to transmit CDV to susceptible hosts. Therefore, CDV is a common cause of disease and death in dogs, ferrets, minks and foxes in Spain (21). This is in agreement with the results of this study in which we found a relatively high CDV seropositive rate in Spanish domestic cats when compared to the situation in Switzerland. These results suggest that viral transmission from dogs to cats, which was demonstrated in a previous experiment (2), is a common event also under natural conditions. This could have been the case in one Swiss cat, which was kept in the same household as an acutely infected dog that eventually died as a consequence of clinical distemper. This cat showed a high antibody titer in the IFA and in the serumneutralization test confirming seroconversion to CDV. Interestingly, no antibodies were found in a second cat living in the same household, which had even closer contact to the dog by sleeping at the same place and eating from the same dish.

The highest seroprevalence of CDV in cats was observed among the samples collected in Abu Dhabi, United Arab Emirates. Of note, there is a low proportion of feral dogs due to religious reasons in this country and clinical distemper is therefore rarely seen. However, there is an estimated large population of feral cats. Two possible reasons could explain the large number of CDV seropositive cats in this country. The first is the presence of another source of CDV than the dog. As the virus is not very stable in the environment, relatively close contact between carrier animals and susceptible hosts is necessary. In the absence of dogs, free-ranging small carnivores, such as

foxes and martens, are possible carrier candidates and could play an important role in the transmission of CDV to feral cats. The second possibility relies on the fact that CDV or a closely related virus could circulate in the cat population independent of external carriers. In this case, cats would also have to shed virus during the acute infection as observed in the dog population. This, however, has not been shown yet and therefore remains a speculation.

There was a lack of virus neutralizing antibodies in most seropositive cats in this study. However, the OP vaccinated cats and the Serengeti lions, which were infected with a CDV strain closely related to OP (29), all had virus neutralizing antibodies against OP. As the CDV field strains are genetically distinct from the vaccine strains, we performed a second virus neutralizing assay using A75/17, but there was just one additional domestic cat with neutralizing antibodies against this field strain. Since wild-type CDV strains differ according to geographical distribution and A75/17 was isolated in America (32), the lack of virusneutralizing antibodies still can be explained by the infection with a different CDV strain or even with another morbillivirus that induced the production of cross-reacting antibodies.

CDV seropositive cats in our study were not at a higher risk of any disease than seronegative cats. In addition, no correlation between sex or age of the cats and the presence of antibodies against CDV could be found. However, the proportion of CDV seropositive cats among the cats from Spain and the United Arab Emirates was significantly higher in the FIV seropositive population than in the FIV seronegative cats. The reasons for that remain unclear. A possible explanation may lie in the fact that several lymphocyte populations are activated especially during early FIV infection (20). This could lead to an increased expression of SLAM (24) resulting in an increased susceptibility to morbillivirus infections. Therefore, FIV and CDV infection may be causally related but do not necessarily correlate with clinical signs. Another explanation would be a higher susceptibility to CDV infection due to

immunosuppression at a late stage of FIV infection. As both FIV and CDV are not very stable in the environment similar transmission routes could also explain the combined occurrence of FIV and CDV infections. Interestingly, in large felids, no correlation between FIV and CDV infection was found. The FIV seropositive lions from the Serengeti National Park were neither at a higher risk of acquiring CDV infection nor was their mortality higher (28, 29). Studies on CDV infected captive large felids in North America and Switzerland did not reveal any association of CDV infection with FIV either (4, 26). This could be explained by the marked genetic heterogeneity and the completely different pathogenicity of the large felid lentiviruses compared to FIV in domestic cats (31, 35). The high rate of CDV seropositive captive large felids in European zoos observed in our study is in accordance with previous reports (14, 26). The source of infection for these captive animals is unknown, but infected free-ranging small carnivores might play an important role in transmission.

In conclusion, CDV infection in domestic cats or infection with a related morbillivirus seems to occur worldwide. In our study, we showed that the seroprevalence in different countries varied from 2% to 18.3% and that dogs possibly play an important role in transmitting virus to susceptible cats. Additional studies investigating virus shedding by cats will be necessary to evaluate the role of cats as carriers. In agreement with previous studies we did not find any clinical symptoms that could be related to CDV infection in seropositive cats. Future studies on the underlying mechanisms will help for a better understanding of the CDV pathogenesis and could lead to new ideas of protection possibilities against clinical distemper. Nevertheless, disease caused by CDV in domestic cats, especially in FIV seropositive individuals at a late stage of infection or in cats with other forms of immune suppression has to be considered.

7 Figures and Tables

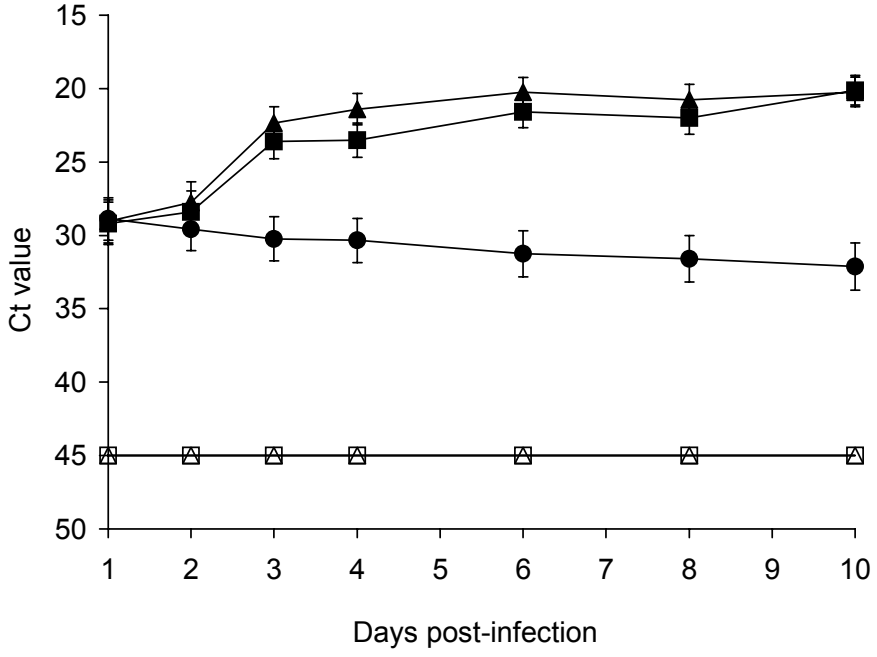

FIGURE 1. Infection of dog and cat PBMC with A75/17 in vitro.

The graph represents the results of a real-time RT-PCR performed using RNA isolated from supernatants of infected dog (▲) and cat (■) PBMC and from cell culture medium incubated with CDV strain A75/17 without cells (●). The results were obtained using a virus dilution of 1:4, but were similar at all other dilutions. Non-infected control dog (Δ) and cat (□) PBMC resulted in a negative signal in the real-time RT-PCR. Results are shown in cycle threshold (Ct) values with their respective standard deviation.

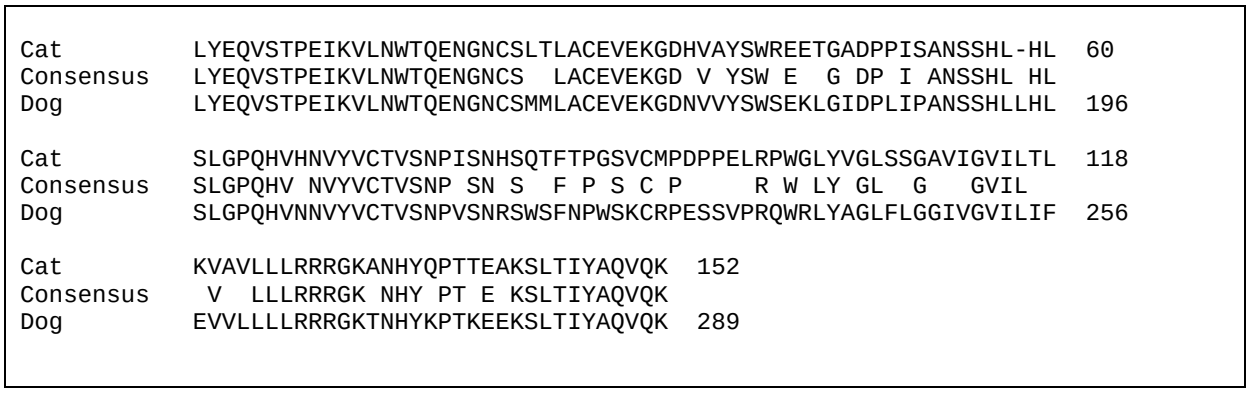

FIGURE 2. Alignement of predicted feline SLAM protein sequence with canine SLAM.

Comparison of deduced amino acid sequence of feline SLAM (BLASTX 2.2.13, Matrix (BLOSUM62), Gap costs (Existence: 11; Extension:1) with dog amino acid sequence. Dashes (-) show gaps of the feline amino acid sequence in comparison to the canine sequence. Consensus shows identical amino acids.

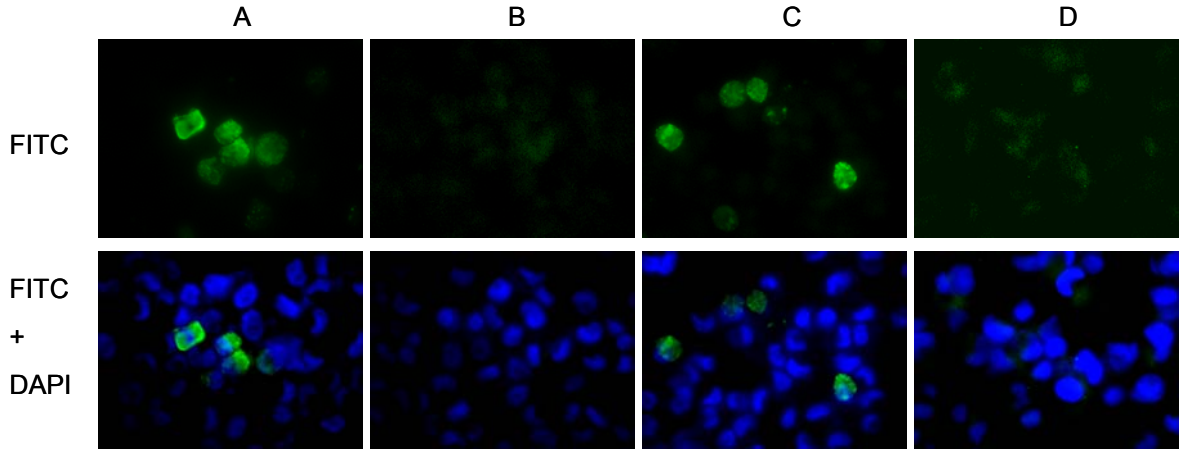

FIGURE 3. Indirect immunofluorescence assay for detection of antibodies specific for the N protein of CDV.

Transfected Vero cells expressing the N protein of CDV strain A75/17 were used as antigen. Panels A represent transfected cells incubated with mouse monoclonal antibody specific for the N protein and secondary FITC-conjugated anti-mouse IgG antibody; panels B represent non-transfected cells incubated with the same antibodies as panels A (negative control for transfection); panels C and D show transfected cells incubated with serum of either a CDV seropositive cat (cat C1 that lived together with a dog with clinical distemper, panels C) or a CDV seronegative cat (SPF cat, panels D) and secondary FITC-conjugated anti-cat IgG antibody. Cat sera were diluted 1:160. Upper panels show the FITC fluorescence, lower panels both the FITC and DAPI fluorescence.

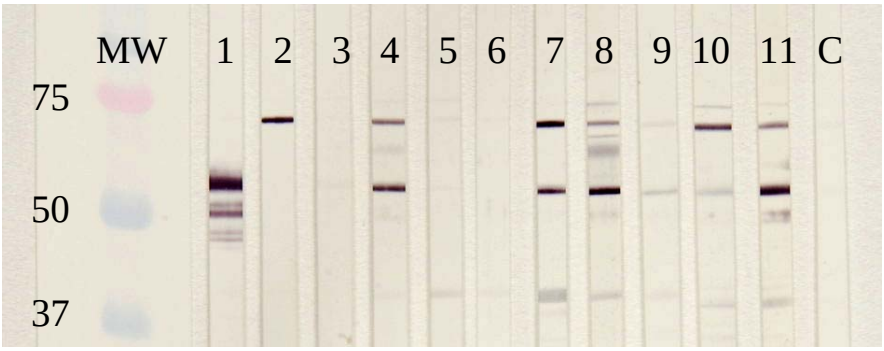

FIGURE 4. Western blot analysis of felid sera reacting with CDV-specific proteins.

lane 1, monoclonal antibody D110 against the N protein; lane 2, monoclonal antibody XI-23 against the H protein; lane 3, cat serum before CDV immunization; lane 4, cat serum after CDV immunization; lanes 5 and 6, SPF cat sera (negative controls); lanes 7-9, representative sera of CDV seropositive domestic cats; lane 10, serum of a healthy non-vaccinated captive liger from Germany, lane 11, serum of a lion from Tanzania with clinical distemper. MW, Molecular weight marker (kDa); C, Control using only secondary antibody.

TABLE 1. Sequence identities of feline SLAM to other species.

	Species	Dog	Cattle	Human	Cotton-headed tamarin	Mouse	Rat
Amino acid	Accession Nr.	NP_001003084	NP_776609	NP_003028	AAG18445	NP_038758	XP_573513
	Identities to feline predicted sequence[1]	72%	68%	66%	64%	54%	53%
Nucleotide	Accession Nr.	AF325357	AF329970	AY0400554	AF302038	AF149791	XP_573513
	Identities to feline sequence[2]	84%	77%	80%	79%	71%	70%

[1] BLASTX 2.2.13, Matrix (BLOSUM62), Gap costs (Existence: 11; Extension:1)

[2] GCG Bestfit, gap creation penalty (50), gap extension penalty (3)

TABLE 2. Comparison of immunofluorescence and western blot with serum neutralization test results.

Seropositive samples[1]	OP virus neutralizing antibodies (titer)		A75/17 virus neutralizing antibodies (ct value difference)	
Domestic cats				
Switzerland	1/10	(1:269)	2/10	(3.16, 6.98)
Spain	2/8	(1:80, 1:135)	2/8	(3.15, 8.64)
UAE	0/17		0/17	
Vaccinated cats	2/2	(1:640, 1:1280)	2/2	(3.89, 4.19)
Large felids				
Free-ranging lions Tanzania	6/6	(1:320, 1:381, 1:453, 1:453, 1:538, 1:538)	3/6	(3.09, 3.58, 3.86)
Captive large felids Europe	2/7	(1:640, 1:1280)	2/7	(3.01, 3.45)

[1] Seropositivity was determined by an indirect immunofluorescence and a Western blot assay. All tested seronegative samples (n=46) were negative in both serum neutralization tests and are therefore not included in the table.

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